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Determination of 2-Acetylaminofluorene Adducts by Immunoassay

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Antisera elicited in rabbits were used in radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) to determine femtomole quantities of deoxyguanosin-(8-yl)-acetylaminofluorene (dg-8-AAF) and deoxyguanosin-(8-yl)-aminofluorene (dg-8-AF). These adducts have been monitored in liver and kidney DNA of male Wistar-Furth rats fed 0.02% or 0.04% 2-acetylaminofluorene (2-AAF) either continuously or for a limited time followed by an interval on control diet. After 24 hr of 0.02% 2-AAF feeding, substantial levels of binding (80 fmole/μg DNA) were observed in liver DNA and increased with time, reaching a plateau of approximately 230 fmole/μg DNA at 30 days and thereafter. During the first week of continuous feeding about 80% of the total C-8 adducts in the liver DNA were deacetylated (dG-8-AF). By 25-60 days, dG-8-AF represented 97-100% of all C-8 adducts as measured by RIA and confirmed by HPLC. Values for C-8 adduct formation in kidney DNA were severalfold lower than in liver and dG-8-AF represented >90% of C-8 adducts at all times studied.

In removal or repair experiments, rats were fed 2-AAF for 3, 7 or 28 days, the 2-AAF diet was discontinued and the liver adducts assayed after intervals on control diet. When dietary 2-AAF administration was for 3 or 7 days, removal of adducts was efficient and almost complete by 28 days on control diet, with preferential retention of dG-8-AF. However, when dietary 2-AAF administration was for 28 days, adduct levels were higher, the repair capacity was saturated and the removal of C-8 adducts was not complete after control diet for a 28-day interval. In a preliminary experiment when [^{3}H]-2-AAF was fed for 3 days, after 25 days of 0.02% 2-AAF, the rates of newly formed adduct formation and removal were similar to those observed for the initial 3 days of 2-AAF feeding. These results demonstrate the predominance and persistence of dG-8-AF in liver and kidney DNA of 2-AAF-fed rats and suggest that the repair capacity of the whole rat liver was not diminished after 1 month of 2-AAF feeding.

Introduction

The development and characterization of highly avid antibodies for carcinogen-DNA adducts has made possible the determination of femtomole ($10^{-15}M$) quantities of such adducts by sensitive immunoassays including radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) (*1*). Antiguanosin-(8-yl)-acetylmainofluorene, elicited in rabbits, has been utilized to quantitate carcinogen-DNA adducts by immunological assays and the results compared favorably to other analytical procedures (*2, 3*). The antiserum is specific of the acetylated and deacetylated C-8 adducts of 2-acetylaminofluorene (2-AAF) with DNA and does not cross-react with the minor adduct, 3-deoxyguanosin-(N^2-yl)-acetylaminofluorene (dG-N^2-AF), the ring-opened adduct 1[6-(2,5-diamino-4-oxypyrimidinyl-N^6-deoxyriboside)]-3-(2-fluorenyl)urea, (diamino Py-Fu) (M. Poirer, unpublished observations), the carcinogen alone or DNA (*4, 5*). The C-8 adducts, *N*-deoxyguanosin-(8-yl)-acetylaminofluorene (dG-8-AAF) and *N*-deoxyguanosin-(8-yl)-aminofluorene (dG-8-AF), comprise the major (approximately 90%) proportion of binding products formed upon interaction of 2- AAF, or its activated derivative *N*-acetoxy-2-acetylaminofluorene (N-Ac-AAF) with DNA *in vivo*, including cultured cells (*6-9*) and whole animals (*6, 10-14*). We have utilized RIA to quantitate formation and removal of guan-8-yl (C-8) adducts in liver and kidney DNA of male rats fed 2-acetylaminofluorene during a period of 2 months. It has been possible not only to deter-

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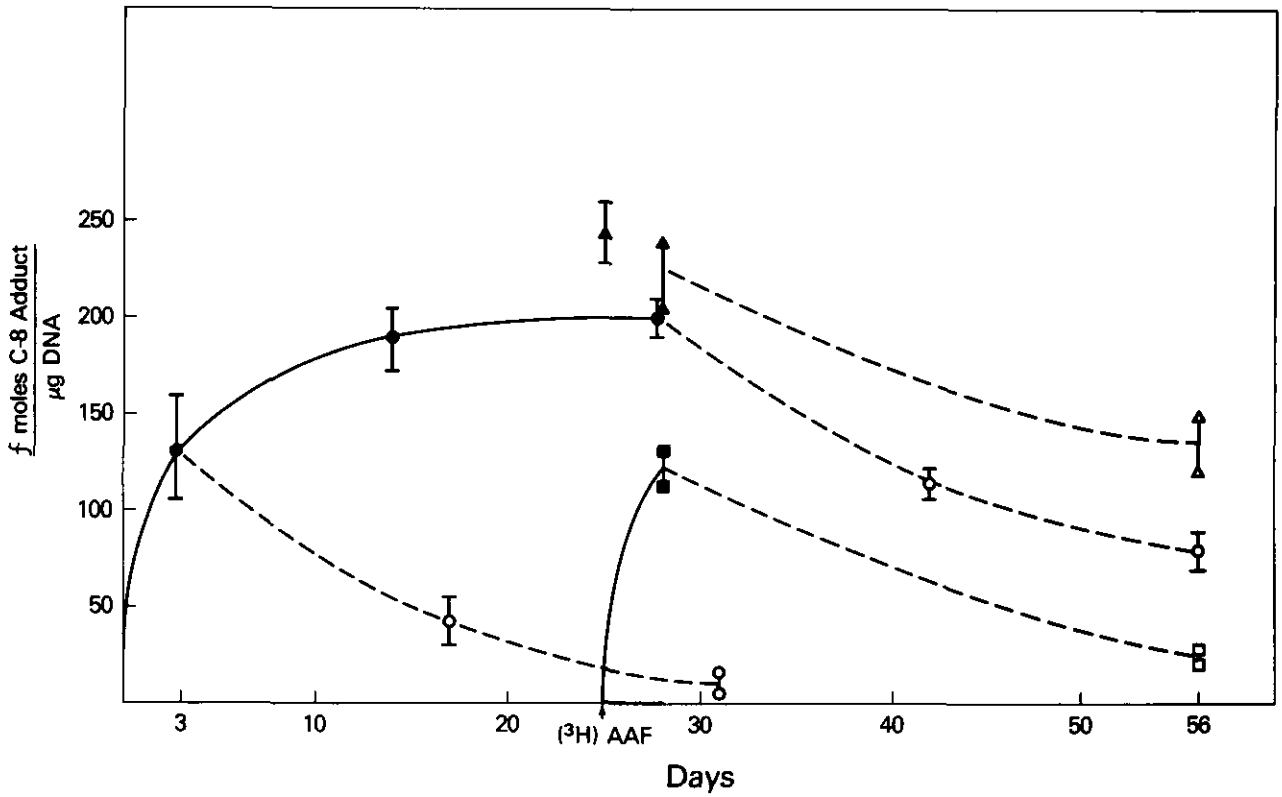

FIGURE 3. Formation (——, ●, ▲, ■) and removal (--, ○, △, □) of C-8 adducts in male WF rats fed 0.02% 2-AAF. Seven animals were fed 0.02% 2-AAF for 25 days, at which time three were sacrificed (▲, 25 days), four were fed 0.02% [^{3}H]-2-AAF (219 mCi/mmole) for 3 days, at which time two were sacrificed (▲, 28 days) and two were given control diet for a subsequent 28 days (△). Values for total C-8 adduct obtained by RIA from these liver DNAs are represented by ▲ and △; values for the newly formed (25-28 days) radioactive adducts in the same animals are represented by ■ and □. In a parallel experiment, values for adduct formation (●) and removal (○) are shown for liver DNA from male WF rats fed 0.02% 2-AAF for 3, 14 and 28 days. DNAs were prepared as indicated for Figure 1, except that P_1 nuclease was used for DNA hydrolysis, and values, represented are either mean ± S.D. for three animals or individual points for two animals.

sent upon cessation of 2-AAF feeding. For example, of the radioactive 2-AAF, only 20% remained after 28 days on control diet when the original adduct level was 120 fmole/μg DNA. Of the total C-8 adduct in the same animals (measured by RIA) 61% remained, from an original adduct level of 220 fmole/μg DNA. Yet both groups of animals had removed about 100 fmole/μg DNA during this time.

Kidney DNAs were assayed from the same animals shown in Figure 3, and the results (not shown) exactly paralleled the data from liver except that the maximum plateau values for C-8 adducts in kidney were 62 fmole/μg DNA.

Proportions of Acetylated and Deacetylated C-8 Adducts in Liver DNA During Feeding of 2-AAF With or Without a Subsequent Interval on Control Diet

Significant differences were observed in the proportions of the acetylated and deacetylated C-8 adducts at various times after the initiation of 2-AAF feeding. For both 0.02% and 0.04% 2-AAF at days 1, 3, 5 and 7, the DNA profiles with [^{3}H]G-8-AAF, and comparison with standard curves indicated that about 20% of the C-8 adducts were acetylated and 80% were deacetylated (Table 1 and Fig. 4). With continuous feeding there was clearly a progression toward a higher proportion of deacetylated C-8 adduct, since liver DNA from animals fed for 25 and 60 days all showed saturation profiles similar to dG-8-AF and hydrolyzed AF-DNA assayed simultaneously (Fig. 4). DNAs which saturate at the same percentage inhibition as the deacetylated standards have been shown to be totally deacetylated, and DNAs which saturate slightly (5-7% inhibition) higher than dG-8-AF in the same assay have been shown by linear regression analysis to contain 3% dG-8-AAF and 97% dG-8-AF (*19, 20*). In liver DNAs from rats fed 2-AAF for 25-60 days 0-3% of the C-8 adduct was the acetylated form (Table 1 and Fig. 4)

Table 1. Proportions of acetylated and deacetylated adducts in rat liver DNA.

Days of feeding		Adducts, %	
AAF	Control diet	dG-8-AF	dG-8-AAF
0.02% AAF			
3	0	80	20
15	0	80	20
25-31	0	97-100	0-3
60	0	97	3
0.04% AAF			
3	0	80	20
15	0	97	3
46	0	97	3
0.02 or 0.04% AAF			
7	7	100	–
7	28	100	–
28	7	97-100	0-3
28	28	97-100	0-3

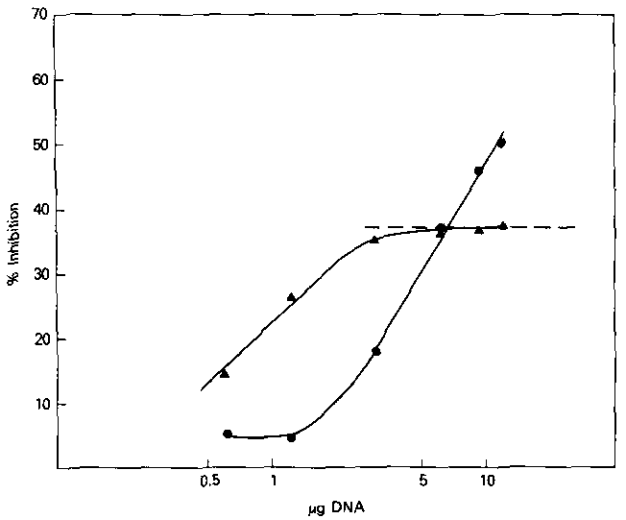

FIGURE 4. DNA profiles in RIA of increasing concentrations of liver DNA samples from male WF rats fed 0.02% 2-AAF for (●) 3 days or (▲) 7 days (--) percent inhibition for standard dG-8-AF saturation in the same assay is indicated. Samples were prepared as indicated in Figure 1 except P_1 nuclease digestion was employed without denaturation for DNA hydrolysis.

and 97-100% was dG-8-AF. Similar criteria indicated that the liver DNAs from rats fed 2-AAF followed by 1-4 weeks of feeding control diet contained 97-100% of C-8 adducts as deacetylated (Table 1).

The experiment (Fig. 3) in which rats were fed [^{3}H]2-AAF for 3 days after 25 days of unlabeled 2-AAF, provided a unique opportunity to compare analysis of adduct formation by RIA and by HPLC. RIA profiles indicated that DNAs from animals fed 2-AAF for 28 days contained dG-8-AF as 97-100% of the total C-8 adduct (Table 1). The HPLC confirmed the presence of one major C-8 adduct peak dG-8-AF, (Fig. 5), and only 1.8-2.9% of the total C-8 radioactivity cochromatographed with the dG-8-AAF standard. In addition there was no evidence for significant quantities of dG-N^2-AAF or diamino-Py-FU in this profile. After 25 days on control diet there was only the dG-8-AF peak which was, this time, much reduced in size (Fig. 5).

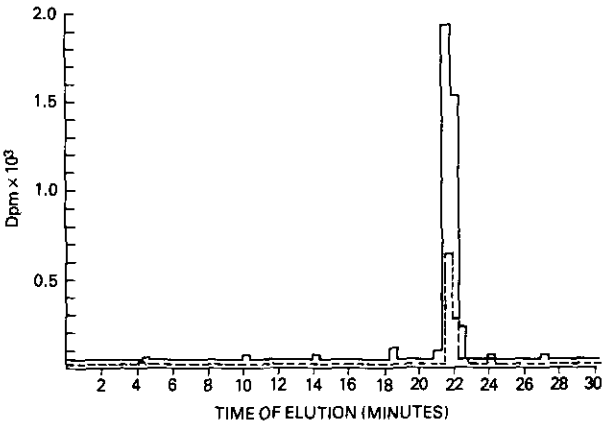

FIGURE 5. High pressure liquid chromatography (HPLC) profiles of radioactivity in liver DNA from male WF rats (– –) fed 0.02% 2-AAF for 25 days followed by 0.02% [^{3}H]-2-AAF (219 mCi/mmole) for 3 days) (--) Parallel animals (--) were additionally fed control diet for 28 days subsequently. DNAs were hydrolyzed with DNAse, alkaline phosphatase and venom phosphodiesterase, and hydrolysis was 90% complete. The largest peak in both profiles eluted in the same position as standard dG-8-AF adduct (22 min). In the experimental profile (——), the small peak at 18.3 min corresponded to the elution of standard dG-8-AAF or G-8-AF. Since this DNA was prepared by CsCl gradient centrifugation this is probably primarily the deoxy adduct. The base line for the control profile (--) was plotted lower by 25 DPM for better resolution of the two lines.

Discussion

Determination of guan-8-yl adducts of 2-acetylaminofluorene in liver and kidney DNAs of rats fed the parent carcinogen has been accomplished utilizing sensitive RIAs with a specific G-8-AAF antiserum, and analytical HPLC after hydrolysis of radioactive DNAs. The combination of both procedures has yielded new information concerning the formation and removal of 2-AAF-DNA adducts in rat liver during 2 months of chronic exposure to a carcinogenic feeding regimen.

Binding levels for total C-8 adducts in the continuous feeding experiments increased rapidly during the first few days of feeding, reaching a plateau above 200 fmole/μg at about 1 month. A similar phenomenon has been observed by Irving (*17*), who fed 0.04% radiolabeled 2-AAF to male rats for 8 weeks and monitored total DNA adducts (including the N^2 adduct which we are unable to measure). Both Irv-

ing (*17*) and Szafartz (*18*) found plateau levels for total adducts in the range of 100-400 fmole/μg DNA upon feeding radioactive 2-AAF or N-OH-AAF at doses between 0.016% and 0.04%. Given the potential sources of variability in carcinogen feeding regimens, it is likely that there is biological significance to these similar plateau values for 2-AAF bound to liver DNA during chronic exposure. In future studies with an initiation-promotion regimen, one could perhaps establish binding levels necessary (but not sufficient) for tumorigenesis.

With short-term feeding or intraperitoneal injection of radiolabeled 2-AAF or N-OH-AAF, several investigators have observed efficient removal of adducts from liver DNA (*10, 12, 18-20*), similar to the data shown in Figure 2 at 3 and 7 days. However, inability of the liver to complete removal of dG-8-AF in DNA after long-term 2-AAF feeding (Fig. 2) has not been previously reported. The experiment with [^{3}H]-2-AAF (Fig. 3) was designed to determine the rate of the newly formed adduct at later times during chronic feeding and also to explore the rate of removal. Even though these results are preliminary and a more extensive time course is needed, these data indicate that the rates of formation and removal of C-8 adducts after 28 days of 0.02% 2-AAF in the diet are similar to those after 3 days of 2-AAF feeding. Thus, after 28 days of 2-AAF feeding there does not appear to be an alteration in removal rates. The adduct levels at 28 days were about 2-fold higher than after 3 days of 2-AAF feeding and the time required for 90% removal at a fixed rate would appear to have been doubled. This experiment, if reproducible, raises many more questions than previously anticipated. It is difficult to reconcile the fact that at both 3 and 28 days the rate of adduct formation was 3- to 4-fold higher than the rate of removal while the adduct levels reach a plateau at approximately 1 month of feeding. Also, how is it possible to incorporate the information that the newly formed adduct constituted half of the C-8 adduct in total liver DNA at 28 days of feeding and appeared to be preferentially removed, since 80% was gone during the removal period? These findings might be explained if binding of carcinogen occurred unevenly in the liver and/or caused cell selection to occur. Such a situation would require that the earliest binding regions would undergo a loss of metabolic capability, thus limiting further adduct formation while still maintaining a fixed removal capacity. In other areas adduct accumulation would occur later during the course of exposure and would have the normal removal capacity. Experiments designed to test this hypothesis will include analysis by immunofluorescence techniques as well as RIA and HPLC.

By RIA we have shown that the deacetylated adduct, dG-8-AF, increased proportionally both with time on the 2-AAF diet, and subsequent feeding of control diet. At early times (1, 3, 5, 7 days) about 80% of the C-8 adduct was deacetylated, and at later times (25-60 days) 97-100% was dG-8-AF. In the repair experiments where animals were fed 2-AAF for 3 or 7 days, there was a marked progression from about 80% dG-8-AF initially to 100% dG-8-AF after a week on control diet. These observations were confirmed by HPLC which showed the presence of one major adduct, dG-8-AF, after 28 days on 0.02% 2-AAF and also after a subsequent 28 days on the control diet. Since Irving has demonstrated a decrease in rat liver sulfotransferase with 2 weeks of 2-AAF feeding (*21*), it was not unexpected to find that neither dG-8-AAF or dG-N^2-AAF were formed in rat liver DNA at this time. Thus it would appear that accumulation of dG-8-AF may be the most persistent sequela of chronic 2-AAF feeding where effects on DNA are concerned. The kinetics of dG-8-AF removal have not been investigated in female rats; the adduct formation follows a pattern similar to that observed in the males (*17*) although dG-8-AF is the only adduct formed (*12*). Since female rats are more resistant to liver tumor formation, presence of the adduct alone probably does not constitute an effect sufficient for tumorigenesis. On the other hand, the acetylated 2-AAF adducts (dG-N^2-AAF and dG-8-AAF) which are formed only in male rats and only at early times during chronic 2-AAF feeding may contribute to the initiating effects of 2-AAF, while accumulation of dG-8-AF may participate somehow in promotion.

In further experiments a combination of immunological and analytical procedures should yield more complete information concerning the accumulation of DNA adducts and the biological responses of the liver during hepatocarcinogenesis by 2-AAF.

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